broadly, to the invention defined in claim 19. Claims 37-46 correspond to claims 20-29. Claim 47 is an alternative to generic claim 36, as explained below.

The objection to the specification is noted. However, section headings are not required. With respect to the remainder of the objection, the specification is amended, hereby, to correct the noted clerical error. The requisite marked up version of the amendment is attached, hereto.

The objection to claim 23 and the rejection of claim 23 under 35 USC 112, ¶2, are overcome by claim 40 replacing claim 23, hereby.

The rejection of claim 18 under 35 USC 112, ¶2, is overcome by claim 36 replacing claim 18, hereby. That is, claim 36 recites the active method step "administering systemically or locally to a body in need thereof."

Claims were rejected un der 35 USC 112, 1st ¶, for allegedly lacking enablement. Reconsideration is requested.

Applicants incorporate herein by reference their remarks addressing the §112, ¶1, rejection set forth in their Amendment filed March 8, 2001.

Moreover, applicants submit amending claim 18 – as claim 36 – concerning the exchange of neuronal regeneration to axonal regeneration renders moot the rejection under §112, ¶1. Moreover, reciting "improvement of the CNS after axonal regeneration" further overcomes the rejection. Additionally, alternative generic claim 47 recites "enhancement of axonal regeneration,' for which enablement exists according to the statement of rejection.

Claims were rejected under 35 USC 102(b) for alleged anticipation based on each of Logan

and Grumet. Claims were rejected under 35 USC 103(a) based on the teachings of Logan in view of White, Krause, and Kivirikko. Reconsideration is respectfully requested.

Applicants incorporate herein by reference their remarks addressing the §102(b)and §103(a) rejections set forth in their Amendment filed March 8, 2001.

With regard to the use to TGF-β the group having published the respective paper indicated that the use of TGF-β does not give any regeneration. WO 93/19783 discloses prevention of a deleterious accumulation of extracellular matrix by contacting the tissue with an agent that inhibits the extracellular matrix producing activity of transforming growth factor-β (TGF-β). The above methods can be used to prevent, suppress or treat scar formation in the CNS. Neutralizing anti-TGF-β-antibodies are considered as useful agents in these methods.

However, in the light of the recent publication by Moon and Fawcett (2001) (copy attached) it has been confirmed that, in contrast to the present invention, treatment of the injured rat brain by neutralizing anti-TGF-β-antibodies does not improve axon regeneration. The observation by Moon and Fawcett (2001) that neutralization of TGF-β reduces astrocytic scar but has no improving effect on axon regeneration can be explained by the broad spectrum of physiological effects of TGF-β. Among these are very beneficial effects of TGF-β such as reducing neuronal cell loss after brain injury (Hughes et al., 1999, copy attached) and promoting re-elongation of injured CNS axons (Abe et al., 1996, copy attached). Therefore, neutralization of TGF-β in CNS lesions at the same time eliminates the beneficial growth-supporting physiological effects of the growth factor. Thus, it is not surprising that this treatment does not promote regeneration. Scientific evidence rather indicates

that neutralization or inhibition of TGF-β as described in WO 93/19783 is counterproductive to CNS regeneration.

In contrast to WO 93/19783 the presently claimed invention related to a method that directly attacks the collagen biosynthesis to inhibit basement membrane formation (leaving TGF-β unaffected). Application of the present invention clearly promotes axon regeneration.

Favorable Action is requested.

Respectfully submitted,

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Marked Up Version of Amendments

Please substitute the following paragraph for page 12, paragraph 2.

--**Electrophysiology and biocytin injections.** Sagittal slices of 400 [m] μm thickness were cut on a vibratome and maintained at 34-35 °C in an interface-type recording chamber. Artificial cerebrospinal fluid (ACSF) consisted of (in mM) 124 NaCl, 3 KCl, 1.25 NaH2PO4, 1.8 MgSO4, 1.6 CaCl2, 26 NaHCO3 and 10 glucose with a pH of 7.4 when saturated with 95% O_2 - 5% CO_2. Stimuli of 100 μs, 5-20 V were delivered via a bipolar tungsten electrode. Extracellular action potentials were registered with a recording electrode (3-5 MW) located in the middle of the postcommissural fornix. Tetrodotoxin (TTX, Sigma) was applied locally in a concentration of 10 μM (dissolved in ACSF) with a broken micropipette placed on the slice surface near the recording site. Injections of a small biocytin (Sigma) crystal into the fornix were performed with a miniature needle. After an incubation period of 8-10 h in the interface chamber, slices were fixed in 4 % paraformaldehyde, resectioned and reacted with ABC peroxidase reagent (Vector Labs). --

CITED REFERENCES

Abe, K., Chu, P.J., Ishihara, A. And Saito, H. (1996)

Transforming growth factor-beta 1 promotes re-elongation of injured axons of cultured rat hippocampal neurons. Brain res. 723, 206-209.

Hughs, P.E., Alexi, T., Walton, M., Williams, C.E., Dragunow, M., Clark, R.G. and Gluckman, P.D. (1999)

Activity and injury-dependent expression of inducible transcription factors, growth factors and apoptosis-related genes within the central nervous system. Prog. Neurobiol. 57,421-450.

Moon, .L.D.F. and Fawcett, J.W. (2001)

Reduction in CNS scar formation without concomitant increase in axon regeneration following treatment of adult rat brain with a combination of antibodies to TGF-β1 and β2. Eur. J. Neuroscience 14, 1667-1677.

ELSEVIER

Brain Research 723 (1996) 206–209

BRAIN RESEARCH

Short communication

Transforming growth factor-β1 promotes re-elongation of injured axons of cultured rat hippocampal neurons

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Accepted 13 February 1996

Abstract

Transforming growth factor-β1 (TGF-β1) is highly expressed in neural tissues following brain injuries. To determine a role of TGF-β1 in neural pathologies, axons of cultured rat hippocampal neurons were cut by local irradiation of laser beam, and the effect of TGF-β1 on neurite regeneration following axotomy was investigated. The axonal growth was stopped by laser irradiation, but addition of TGF-β1 remarkably promoted the axonal re-elongation from the injured site. The effect of TGF-β1 was concentration dependent and seen maximally at a concentration of 1 ng/ml. These results suggest that TGF-β1 has a capability of promoting axonal regeneration of brain neurons after lesioning.

Keywords: Transforming growth factor-β1; Neurite regeneration; Cultured hippocampal neuron

Transforming growth factor-β (TGF-β) is a multifunctional regulatory peptide produced by a variety of normal and transformed cell in vitro and in vivo [14,22]. It was first characterized as promoting the anchorage-independent growth of fibroblast [4], but has also been reported to regulate embryogenesis [7,19], formation of extracellular matrix [2] and growth and differentiation of many types of cells [23]. The family of TGF-βs at present comprises five distinct but highly homologous isoforms, and only TGF-β1, -β2 and -β3 are found in mammals [16,25].

TGF-β1 is not expressed at significant levels in normal neural tissues, but seen at high levels under pathological conditions. For example, immunoreactive TGF-β1 is seen in macrophages and astrocytes within neural tissue infected with HIV-1 [24]. After mechanical lesioning [10,13,18,24] and hypoxic ischemia [9], TGF-β1 is rapidly and transiently expressed within local damaged neural tissue. These in vivo observations suggest a role of TGF-β1 in brain pathologies. The effect of TGF-β1 on cultured brain neurons has been very little studied, but we have recently found that TGF-β1 promotes neurite sprouting and elongation of cultured rat hippocampal neurons [8]. Whether or not TGF-β1 exerts the growth-promoting effect on damaged neurons is an important subject to clarify a role of TGF-β1 in neural pathologies. We have recently developed a simple assay system in which the growth cone of the axon of cultured neurons was injured by local irradiation of laser beam and the effects of reagents were evaluated for axonal re-elongation or other morphological changes in the partially damaged neurons [3,17]. By using this assay system, in the present study, we investigated the effect of TGF-β1 on neurite regeneration of injured brain neurons in vitro.

We used the ACAS 470 Work Station (Meridian) to memorize the cell position on the microscope stage and to deliver Argon laser beam (488 nm) [3]. Dissociated hippocampal neurons were prepared from 18-day-old embryos of Wistar rats as described in our previous paper [1] and plated on polylysine-coated culture dish at a density of 2500 cells/cm^2. The culture dish was prepared from a 35-mm Petri dish by attaching a special glass coverslip to the inner surface with silicone grease [3]. The special glass coverslip was sealed with a film that absorbs laser beam and converts laser beam to heat. When laser beam was irradiated, the neurites were virtually damaged by heat. After incubation in serum-containing medium for 24 h, the medium was changed to serum-free modified Eagle's medium [3] and the cells were cultured for a further 24 h. During this preliminary culture period, hippocampal neurons acquired their stereotypical morphology with one long process and several short processes. By morphological and

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immunocytological criteria, the long process and several short processes have been identified as axon and dendrites, respectively [5,15]. The term 'neurite' is used to include both axons and dendrites. The hippocampal pyramidal neurons that had established axons and dendrites were selected, and the laser beam (output 40 mW, acoust-optic-modulator 35%, 1 s) was delivered to the axonal growth cone. This moderate condition of laser irradiation did not significantly affect the survival of neurons [3]. The cells were photographed immediately after laser irradiation, and then TGF-β1 was added to the culture medium. The same cells were photographed 24 and 48 h after addition of TGF-β1. Measurement of morphological parameters was made by tracing the photographs on a digitizing tablet.

TGF-β1 from human platelets (Wako Pure Chemical Industries, Osaka, Japan) was reconstituted in 20 μl of 0.1 M acetate and then diluted to a concentration of 1.5 μg/ml in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline supplemented with 1 mg/ml bovine serum albumin. It was further diluted to desired concentrations by adding the culture medium immediately before application in cell cultures. In preliminary experiments, bovine serum albumin alone showed no significant effect on neuronal survival and morphology at concentrations up to 10 μg/ml.

First, the influence of laser irradiation was checked in control cultures. Following laser irradiation, the axonal elongation ceased, but there were no changes in the growth of branches at proximal part of axon, the number of neurites per soma and the dendritic growth (Fig. 1A), consistent with our previous observation [3].

Next the effects of TGF-β1 on the morphological changes following axonal lesioning were investigated. Since the distance of the injured site from the soma may influence the regenerative response of neurons [6], we confirmed that the axon length from the soma to the injured site was not significantly different among the tested groups (Fig. 2A). Fig. 1B shows micrographs of a representative neuron cultured in the presence of 1 ng/ml TGF-β1. The results of quantitative analyses in many neurons are shown in Fig. 2B–H. TGF-β1 greatly promoted the re-elongation of axons from the injured site (Fig. 2B and C). No significant change in branch points in regenerated axons (Fig. 2D) means that injured axons extended without bifurcating. We have previously observed that basic fibroblast growth factor (bFGF) promotes the branching of proximal part of injured axon [3]. However, TGF-β1, unlike bFGF, did not affect the branching in proximal part of injured axons (Fig. 2E and F). The number of primary neurites emanating from the soma (Fig. 2G) and total length of uninjured dendrites (Fig. 2H) were not affected by TGF-β1. The axonal regeneration-promoting effect of TGF-β1 was seen maximally at 1 ng/ml, but declined at a higher concentration (10 ng/ml).

The main finding in the present study is that TGF-β1 is very effective in promoting re-elongation of cut axons of cultured brain neurons. Since the moderate condition of laser irradiation employed in the present study does not affect the neuronal survival and since TGF-β1 has little effect on the survival of cultured rat hippocampal neurons [8], the promotion of axonal regeneration by TGF-β1 is independent of neuronal survival and probably reflects its direct action on growth-regulating mechanisms. Furthermore, the re-elongation of injured axons of neurons which once grew and differentiated in culture is different from

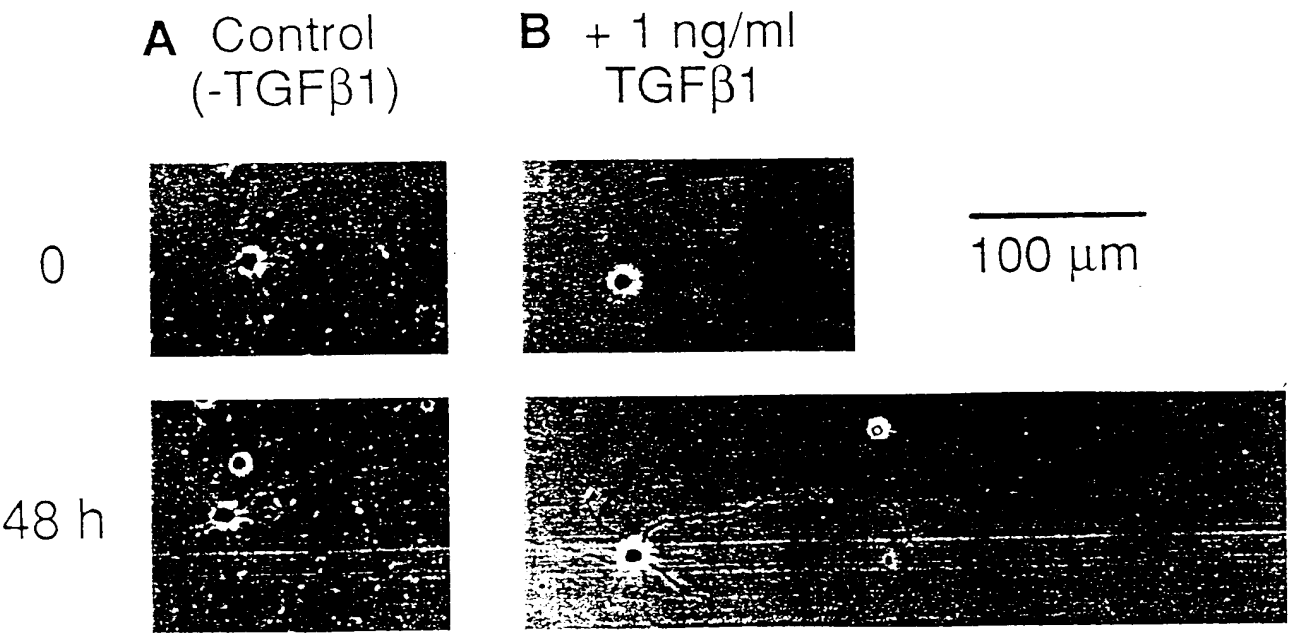

Fig. 1. Representative photomicrographs showing cultured rat hippocampal neurons immediately following axotomy (0 h) and 48 h after axotomy in the control medium (A) or in the medium with added 1 ng/ml TGF-β1 (B). Arrows indicate the sites injured by laser irradiation.

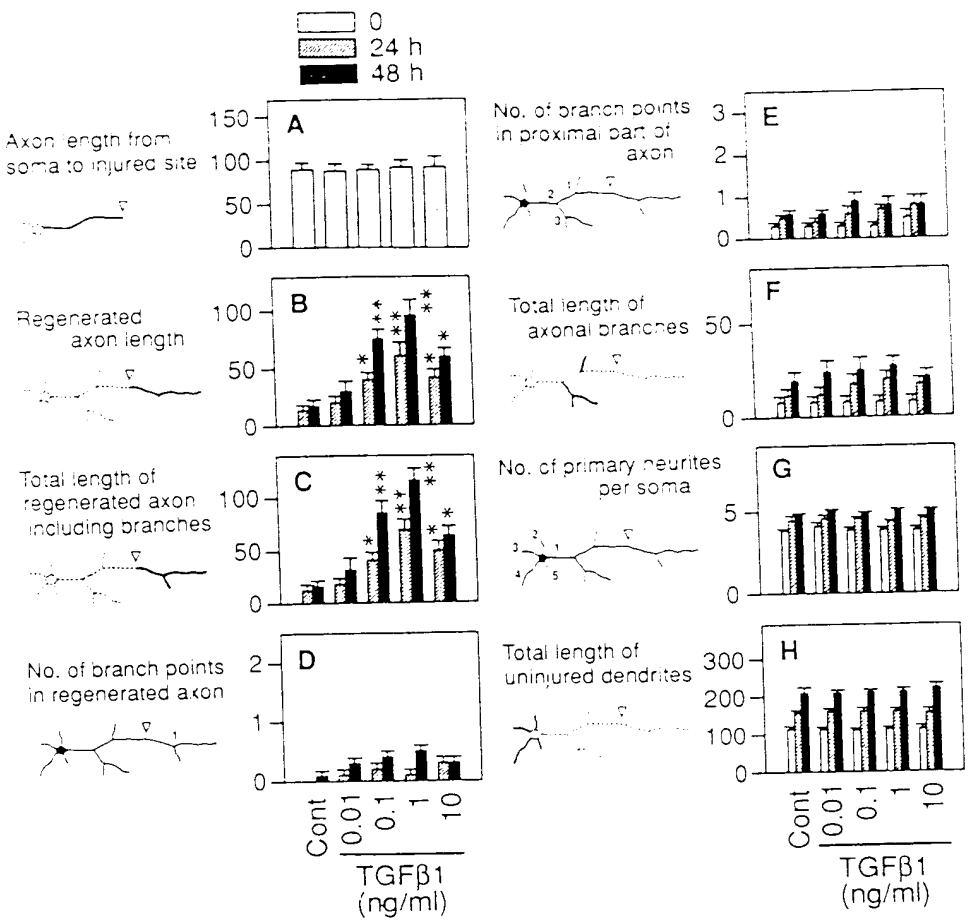

Fig. 2. The effect of TGF-β1 on morphology of cultured rat hippocampal neurons following axonal lesioning. The cells were photographed immediately after (0 h, white columns), 24 (shaded columns) and 48 h (solid black columns) after axonal lesioning and addition of TGF-β1 (0.01–10 ng/ml). Definition of each morphological parameter is illustrated on the left side of graphs. First (A) the axon length from soma to injured site was measured from the photographs taken immediately after laser irradiation (0 h), and it was confirmed that this value was not different among the tested groups. (B) Regenerated axon length from injured site, (C) total length of regenerated axon including branches and (D) the number of branch points in regenerated axon were measured as indicators of axonal regeneration from the lesioned site. Changes in branches at proximal part of injured axons were quantitated by measuring (E) the number of branch points and (F) the total length of axonal branches. Changes in uninjured neurites (dendrites) were quantitated by measuring (G) the number of primary neurites directly emanating from the soma and (H) the total length of dendrites. White arrow heads indicate the site injured by laser irradiation. The numbers of neurites and branch points were counted as illustrated. Neurite lengths corresponded to the lengths of lines drawn in bold strokes. The data are represented as the means and S.E.M. ($n = 30$ cells). Asterisks indicate significant differences from the values in control group (Cont) which received axonal lesions but was not treated with TGF-β1: * $P < 0.05$, ** $P < 0.01$; Dunnett's test.

the initial phase of growth of dissociated neurons in normal culture conditions. For example, interleukin-2 promotes neurite elongation of cultured rat hippocampal neurons [21] but does not affect the re-elongation of cut axons [our unpublished observation]. Therefore, the present result suggests that TGF-β1 has a capability of promoting axonal regeneration of brain neurons after lesioning.

The reason why the regeneration-promoting effect of TGF-β1 declined at a higher concentration (10 ng/ml) is not clear, but some possibilities can be considered: (1) an unknown inhibitory compound may be present as a minor contaminant in the preparation; (2) TGF-β may have not only stimulatory but also inhibitory effects; (3) TGF-β receptor or TGF-β-stimulated signal transduction mechanisms may be down-regulated by exposure to a high concentration of TGF-β1.

It has been reported that, within damaged neural tissue, TGF-β1 plays a primary role in the mesodermal scar formation by activating invasion of macrophages and fibroblasts [20]. The scar formation after brain injury is necessary but paradoxically not helpful to the reconnection of neural pathways. Since physical barrier of scar material produced by TGF-β1 possibly limits regeneration of damaged axons, several groups have tried to test neutralizing antibodies to TGF-β1 to inhibit scar formation and promote neuronal regeneration [11]. However, neural regeneration was not enhanced in scar-inhibited wounds [12]. We found in the present study that TGF-β1 promotes axonal

regeneration of damaged neurons. The failure of the previous therapeutic trials with TGF-β1 antibodies may be due to that the antibodies inhibit not only scar formation but also neuronal regeneration supported by TGF-β1. It is necessary to distinguish cellular mechanisms underlying TGF-β1-mediated scar formation and neuronal regeneration.

In conclusion, we have shown for the first time that TGF-β1 has a capability of promoting the axonal re-elongation of injured brain neurons. It is possible that TGF-β1 expressed in injured neural tissue after lesioning or ischemia [9,10,13,18] play a role in promoting neural regeneration and reconnection of neural networks.

References

[1] Abe, K., Takayanagi, M. and Saito, H., Effects of recombinant human basic fibroblast growth factor and its modified protein CS23 on survival of primary cultured neurons from various regions of fetal rat brain, *Jpn. J. Pharmacol.*, 53 (1990) 221–227.

[2] Bastois, A. and Massague, J., Transforming growth factor β regulate the expression and structure of extracellular matrix chondroitin/dermatan sulfate proteoglycans, *J. Biol. Chem.*, 263 (1988) 3039–3045.

[3] Chu, P.-J., Saito, H. and Abe, K., Polyamines promote regeneration of injured axons of cultured rat hippocampal neurons, *Brain Res.*, 672 (1995) 233–241.

[4] De Larco, J.E. and Todaro, G.J., Growth factors from murine sarcoma virus-transformed cells, *Proc. Natl. Acad. Sci. USA*, 75 (1978) 4001–4115.

[5] Dotti, C.G., Sullivan, C.A. and Banker, G.A., The establishment of polarity by hippocampal neurons in culture, *J. Neurosci.*, 8 (1988) 1454–1468.

[6] Goslin, K. and Banker, G., Experimental observations on the development of polarity by hippocampal neurons in culture, *J. Cell Biol.*, 108 (1989) 1507–1516.

[7] Heine, U.I., Muroz, E.F., Flanders, K.C., Ellingsworth, L.R., Lam, H.Y.P., Thompson, N.C., Roberts, A.B. and Sporn, M.B., Role of transforming growth factor-β in the development of the mouse embryo, *J. Cell Biol.*, 105 (1987) 2861–2876.

[8] Ishihara, A., Saito, H. and Abe, K., Transforming growth factor-β1 and -β2 promote neurite sprouting and elongation of cultured rat hippocampal neurons, *Brain Res.*, 639 (1994) 21–25.

[9] Klempt, N.D., Sirimanne, E., Gunn, A.J., Klempt, M., Singh, K., Williams, C. and Gluckman, P.D., Hypoxia-ischemia induces transforming growth factor-β1 mRNA in the infant rat brain, *Mol. Brain Res.*, 13 (1992) 93–101.

[10] Lindholm, D., Castren, E., Kiefer, R., Zafra, F. and Thoenen, H., Transforming growth factor-β1 in the rat brain: increase after injury and inhibition of astrocyte proliferation, *J. Cell Biol.*, 117 (1992) 395–400.

[11] Logan, A. and Berry, M., Transforming growth factor-β1 and basic fibroblast growth factor in the injured CNS, *Trends Pharmacol. Sci.*, 14 (1993) 337–343.

[12] Logan, A., Berry, M., Gonzalez, A.-M., Frautschy, S.A., Sporn, M.B. and Baird, A., Effects of transforming growth factor β1 on scar production in the injured central nervous system of the rat, *Eur. J. Neurosci.*, 6 (1994) 355–363.

[13] Logan, A., Frautschy, S.A., Gonzalez, A.-M., Sporn, M.B. and Baird, A., Enhanced expression of transforming growth factor-β1 in the rat brain after a localized cerebral injury, *Brain Res.*, 587 (1992) 216–225.

[14] Lyons, R.M. and Moses, H.L., Transforming growth factors and the regulation of cell proliferation, *Eur. J. Biochem.*, 187 (1990) 467–473.

[15] Mattson, M.P., Dou, P. and Kater, S.B., Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons, *J. Neurosci.*, 8 (1986) 2087–2100.

[16] Miller, D.A., Lee, A., Matsui, Y., Chen, E.Y., Moses, H.L. and Derynck, R., Complementary DNA cloning of the murine transforming growth factor β3 (TGFβ3) precursor and the comparative expression of TGF β3 and TGF β1 messenger RNA in murine embryos and adult tissues, *Mol. Endocrinol.*, 3 (1989) 1926–1934.

[17] Miyagawa, T., Saito, H. and Nishiyama, N., Branching enhancement by basic fibroblast growth factor in cut neurite of hippocampal neurons, *Neurosci. Lett.*, 153 (1993) 29–31.

[18] Nichols, N.R., Laping, N.J., Day, J.R. and Finch, C.E., Increases in transforming growth factor-β mRNA in hippocampus during response to entorhinal cortex lesions in intact and adrenalectomized rats, *J. Neurosci. Res.*, 28 (1991) 134–139.

[19] Pelton, R.W., Nomura, S., Moses, H.L. and Hogan, B.L.M., Expression and transforming growth factor-β2 mRNA during murine embryogenesis, *Development*, 106 (1989) 759–767.

[20] Roberts, A.B., McCune, B.K. and Sporn, M.B., TGF-β: regulation of extracellular matrix, *Kidney Int.*, 41 (1992) 557–559.

[21] Sarder, M., Saito, H. and Abe, K., Interleukin-2 promotes survival and neurite extension of cultured neurons from fetal rat brain, *Brain Res.*, 625 (1993) 347–350.

[22] Sporn, M.B., Roberts, A.B., Wakefield, L.M. and De Crombrugghe, B., Some recent advances in the chemistry and biology of transforming growth factor-beta, *J. Cell Biol.*, 105 (1987) 1039–1045.

[23] Tucker, R.F., Shipley, G.D., Moses, H.L. and Holley, R.W., Growth inhibitor from BSC-1 cells is closely related to the platelet type β transforming growth factor, *Science*, 226 (1984) 705–707.

[24] Wahl, S.M., Allen, J.B., McCartney-Francis, N., Morganti-Kossmann, M.C., Kossmann, T., Ellingsworth, L., Mai, U.E., Mergenhagen, S.E. and Orenstein, J.M., Macrophage- and astrocyte-derived transforming growth factor β as a mediator of central nervous system dysfunction in acquired immune deficiency syndrome, *J. Exp. Med.*, 173 (1991) 981–991.

[25] Wilcox, J.N. and Derynck, R., Developmental expression of transforming growth factors alpha and beta in mouse fetus, *Mol. Cell Biol.*, 8 (1988) 3415–3422.

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FULL-TEXT ARTICLE

Activity and injury-dependent expression of inducible transcription factors, growth factors and apoptosis-related genes within the central nervous system.

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This review primarily discusses work that has been performed in our laboratories and that of our direct collaborators and therefore does not represent an exhaustive review of the current literature. Our aim is to further discuss the role that gene expression plays in neuronal plasticity and pathology. In the first part of this review we examine activity-dependent changes in the expression of inducible transcription factors (ITFs) and neurotrophins with long-term potentiation (LTP) and kindling. This work has identified particular ITFs (Krox-20 and Krox-24) and neurotrophin systems (particularly the brain-derived neurotrophic factor (BDNF)/tyrosine receptor kinase-B, Trk-B system) that may be involved in stabilizing long-lasting LTP (i.e. LTP3). We also show that changes in the expression of other ITFs (Fos, Jun-D and Krox-20) and the BDNF/trkB neurotrophin system may play a central role in the development of hippocampal kindling, an animal model of human temporal lobe epilepsy. In the next part of this review we examine changes in gene expression after neuronal injuries (ischemia, prolonged seizure activity and focal brain injury) and after nerve transection (axotomy). We identify apoptosis-related genes (p53, c-Jun, Bax) whose delayed expression selectively increases in degenerating neurons, further suggesting that some forms of neuronal death may involve apoptosis. Moreover, since overexpression of the tumour-suppressor gene p53 induces apoptosis in a wide variety of dividing cell types we speculate that it may perform the same function in post-mitotic neurons following brain injuries. Additionally, we show that neuronal injury is associated with rapid, transient, activity-dependent expression of neurotrophins (BDNF and activinA) in neurons, contrasting with a delayed and more persistent injury-induced expression of certain growth factors (IGF-1 and TGFbeta) in glia. In this section we also describe results linking ITFs and neurotrophic factor expression. Firstly, we show that while BDNF and trkB are induced as immediate-early genes following injury, the injury-induced expression of activinA and trkC may be regulated by ITFs. We also discuss whether loss of

retrograde transport of neurotrophic factors such as nerve growth factor following nerve transection triggers the selective and prolonged expression of c-Jun in axotomized neurons and whether c-Jun is responsible for regeneration or degeneration of these axotomized neurons. In the last section we further examine the role that gene expression may play in memory formation, epileptogenesis and neuronal degeneration, lastly speculating whether the expression of various growth factors after brain injury represents an endogenous neuroprotective response of the brain to injury. Here we discuss our results which show that pharmacological enhancement of this response with exogenous application of IGF-1 or TGF-beta reduces neuronal loss after brain injury.

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- Review, Academic

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Reduction in CNS scar formation without concomitant increase in axon regeneration following treatment of adult rat brain with a combination of antibodies to TGFβ_1 and β_2

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Keywords: central nervous system, glia, regeneration, scar, transforming growth factor β

Abstract

In this study we investigated whether CNS axons regenerate following attenuation of scar formation using a combination of antibodies against two isoforms of transforming growth factor beta (TGFβ). Anaesthetized adult rats were given unilateral mechanical lesions of the nigrostriatal tract. Implantation of transcranial cannulae allowed wounds to be treated with a combination of antibodies against TGFβ_1 and TGFβ_2 once daily for 10 days postaxotomy. Eleven days post-transection brains from animals under terminal anaesthesia were recovered for histological evaluation. Gliosis, inflammation and the response of dopaminergic nigral axons were assessed by immunolabelling. Treatment with antibodies against TGFβ_1 and TGFβ_2 attenuated (but did not abolish) the response of glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes and of NG2-immunoreactive glia but did not attenuate the response of CR3-immunoreactive microglia and macrophages. However, this reduction in scar formation was not accompanied by growth of cut dopaminergic nigral axons. We conclude that treatment of injured adult rat brain with a combination of antibodies against TGFβ_1 and TGFβ_2 results in a reduction of scar formation but that this is not sufficient to enhance spontaneous long distance CNS axon regeneration

Introduction

Following CNS injury, adult mammalian neurons do not regenerate axons through regions of scar formation (Brecknell & Fawcett, 1996). It is, therefore, important to determine whether attenuating scar formation might enhance CNS axon regeneration. Scar formation is regulated in part by different isoforms of transforming growth factor beta (TGFβ) (Logan & Berry, 1993; McCartney-Francis & Wahl, 1994; Krieglstein et al., 1995; Raivich et al., 1999). In particular, following cortical stab injury in adult rats, whereas intraventricular infusion of TGFβ_1 potentiates aspects of scar formation, chronic intraventricular infusion of antagonists to different isoforms of TGFβ reduces several cellular and molecular components of scar formation, leading to reduced astrocytosis and attenuated deposition of laminin and fibronectin. This has been shown using antibodies to TGFβ_1 (Logan et al., 1994), or antibodies to TGFβ_2 (Logan et al., 1999b), or a nonspecific TGFβ antagonist (the chondroitin sulphate proteoglycan), decorin (Logan et al., 1999a). Panspecific antibodies to TGFβ also reduce collagen production in injured peripheral nerve (Nath et al., 1998).

Further evidence that TGFβ isoforms organize the CNS wounding response comes from *in vitro* work showing that TGFβ enhances production of extracellular matrix molecules by astrocytes, oligodendrocyte progenitors and fibroblasts (Varga et al., 1987; Baghdassarian-Chalaye et al., 1993; Flanders et al., 1993; Smith & Hale, 1997; Schnädelbach et al., 1998). For example, *in vitro*, TGFβ enhances production by astrocytes of the chondroitin sulphate proteoglycans, neurocan and versican (Asher et al., 1999, Asher et al. 2000). Thus, TGFβ is known to enhance production of at least two proteoglycans which are abundant at CNS injury sites and which may limit spontaneous CNS axon regeneration.

In this paper we test first whether delivery of a combination of antibodies to TGFβ_1 and TGFβ_2 can reduce injury-evoked gliosis and second, whether any reduction of gliosis is accompanied by an increase in growth of CNS axons. We have performed these experiments in a well-characterized model of CNS axotomy designed for assessment of axon regeneration, namely, following unilateral transection of the nigrostriatal tract in anaesthetized adult rats (Brecknell et al., 1995). We have shown in previous experiments that postinjury modifications of the glial scar environment can promote CNS axon regeneration (Moon et al. 2000; Moon et al. 2001). In the present experiments, we find that administration of antibodies against TGFβ_1 and TGFβ_2 reduces some aspects of glial scarring but does not promote significant CNS axon regeneration.

Materials and methods

Animal care

All animals were treated in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and associated guidelines. Animals were housed in groups on a 12-h light : 12-h dark cycle, lights on during the day, and were given food and water *ad libitum* as

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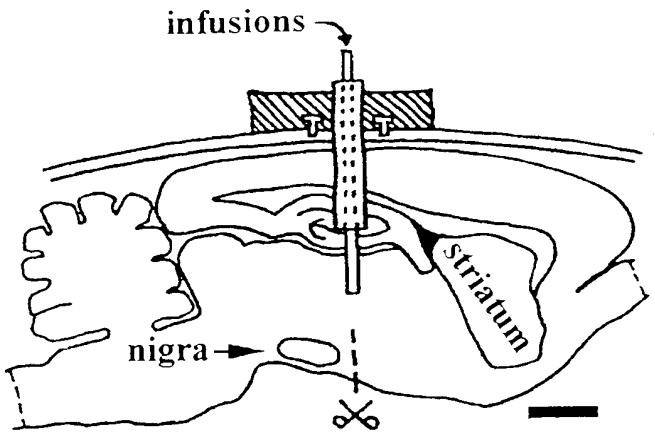


FIG. 1. Parasagittal schematic showing site of transcranial cannulation allowing infusion of solutions into site of unilaterally transected nigrostriatal tract (scissors). Scale bar, 2 mm.

well as playthings to reduce boredom. Following surgery, animals often appeared unkempt due to temporary cessation of grooming and weight loss averaged 20% but both these conditions were reversed within 7–10 days. Animals were handled, inspected and weighed daily. Postoperative diet was supplemented with dog food and wet mash, and to counteract dehydration animals were given 10 mL 4% glucose in 0.18% saline subcutaneously during surgery and thereafter as required. Clean cages were used every 2 days. For analgesia, animals were given soluble paracetamol (1 mg/mL) in their drinking water for at least 3 days postoperatively.

Surgery

Twenty-seven adult Sprague–Dawley rats, weighing 147–223 g, were given unilateral knife cut lesions of the nigrostriatal tract and indwelling cannulae were implanted so as to allow infusion of substance into the lesion site once daily for 10 days postoperatively (Fig. 1).

Rats were anaesthetized using halothane (5%) in carrier (oxygen, 2 L/min) and transferred to a stereotaxic frame (David Kopf Instruments, USA) with the incisor bar set 2.3 mm below the interaural line. Anaesthesia was maintained thereafter with halothane (1.5%–2.0%) in carrier (oxygen, 0.6 L/min) with inhalational analgesic (nitrous oxide, 0.6 L/min). The preshaved scalp was washed with 70% ethanol and painted with antiseptic ointment (Betadine, UK) prior to making a midline incision, retracting the skin and clearing the periosteum from the cranium. A dental drill with size three drill bit was used to remove small pieces of skull where necessary. All stereotaxic coordinates are measured in millimetres with anterior and lateral coordinates made relative to bregma and vertical coordinates made relative to dura. The right medial forebrain bundle (including the nigrostriatal tract) was transected using an extruding wire 'Scouten' knife (David Kopf Instruments, USA) as follows. The tip was lowered to A, −3.0; L, +3.0 and V, −8.0 (according to the atlas of Paxinos & Watson, 1986) and the wire blade was extruded such that it formed a smooth curve in the coronal plane reaching medially to the midline and ventrally to the base of the brain. The assembly was withdrawn vertically by 4 mm and the blade retracted and re-extruded. Finally, the assembly was relowered by 4 mm, the blade was retracted and the entire assembly was withdrawn from the brain. This procedure twice transects the right nigrostriatal tract approximately 650 μm anterior to the substantia nigra and 4 mm posterior to the proximal striatal border (Brecknell et al., 1995).

TABLE 1. Treatment groups and numbers of rats in each

Treatment	Rats per group (n)
Axotomy + sham	3
Axotomy + saline + anti$TGF\beta_1$ + anti$TGF\beta_2$	7
Axotomy + saline + IgG4 control antibodies	8
Axotomy + saline + GDNF	3
Axotomy + saline + GDNF + anti$TGF\beta_1$ + anti$TGF\beta_2$	3
Axotomy + saline + bFGF + IL-1α + anti$TGF\beta_1$ + anti$TGF\beta_2$	3

Immediately following axotomy, an indwelling cannula was secured transcranially to allow administration of substances into the lesion site. Stainless steel tubing (Coopers Needle Works, Birmingham, UK) was used to make indwelling cannulae (length 7 mm, 23 gauge), infusion cannulae (11 mm, 30 gauge, bent to a 45° angle, 4 mm from one end) and occlusion stylets (12 mm, 30 gauge stainless steel, bent to a 45 degree angle 8 mm from one end). Indwelling cannulae were certified free of obstructions prior to use by checking patency with the occlusion stylets. A small hole was drilled to allow placement of the cannula above the lesion site (A, −2.5 and L, −2.5). Stainless steel screws (1.6 mm, Semat Technical UK Ltd) were inserted into each of three drill holes placed around the cannula site to form an equilateral triangle with sides of three millimetres. A 5-mm length of 2 mL syringe barrel (Plastipak, Becton Dickinson, UK) was placed around the screws and through this the indwelling cannulae were stereotactically lowered 3.0 mm below dura such that 3.0 mm protruded above the 1 mm-thick skull. Quick setting acrylic dental cement (Simplex rapid acrylic powder premixed with methyl methacrylate, Associated Dental Products Ltd, Swindon, UK) was poured into the syringe barrel and left to dry for at least 8 mins thereby firmly securing the indwelling cannula to the intracranial screws. A stylet was inserted into the indwelling cannula to occlude the cylinder when not in use. Following surgery, the wound was closed with absorbable sutures (Vicryl 4/0, Ethicon, UK) and antiseptic powder was applied.

On post-transection days 0–10 inclusive, animals received either sham infusions or 3 μL infusions of various combinations of the following substances in 0.9% saline (See Table 1 for combinations): control antibodies (human IgG4, 750 μg/mL, Sigma, UK); recombinant antibodies to $TGF\beta_1$ (anti-$TGF\beta_1$, 250 μg/mL, Cambridge Antibody Technology, Melbourn, Cambridge, UK); recombinant antibodies to $TGF\beta_2$ (anti-$TGF\beta_2$, 500 μg/mL; Cambridge Antibody Technology); recombinant human glial cell line derived neurotrophic factor (GDNF, 83 μg/mL, Amgen, USA); recombinant mouse basic fibroblast growth factor (bFGF, 20 ng/mL, Boehringer, UK) and recombinant mouse interleukin 1α (IL1α, 4 ng/mL, Genzyme, USA). Anti-$TGF\beta_1$ is a fully human, single variable chain fragment antibody while anti-$TGF\beta_2$ is a fully human, whole IgG4 antibody. The ability of these anti-TGFβ antibodies to bind and neutralize particular isoforms of TGFβ have been described previously (Logan et al., 1994; Logan et al., 1999b). Aliquots of stock solutions were kept frozen at −70 °C and thawed to room temperature immediately prior to use.

Infusion cannulae were made from stainless steel tubing (11 mm, 30 gauge) bent to a 45° angle, 4 mm from one end, with the short arm connected via 30 cm of clear polythene tubing (0.28 mm internal diameter, 0.61 mm external diameter, Portex, UK) to a 10-μL syringe

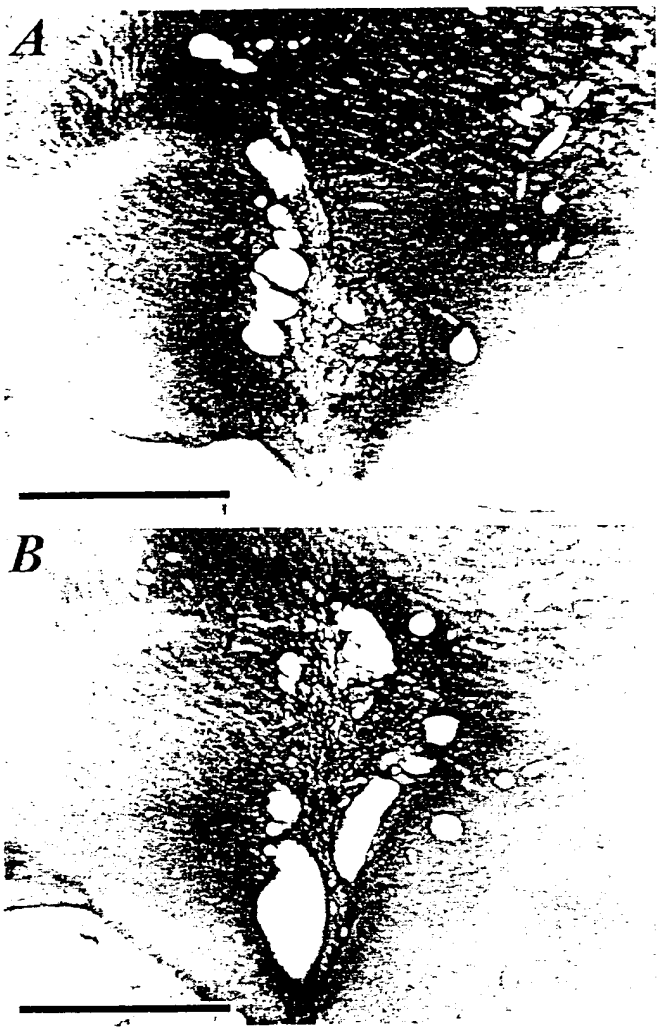


FIG. 2. Immunolabelling using anti-human antibodies 11 days following transection, i.e. 1 day following the eleventh infusion of either (A) human IgG4 control antibodies or (B) human antibodies against TGFβ1 and TGFβ2. Immunolabelling was present within the lesion core and in a 300-μm-thick rim surrounding the lesion core, confirming effective perilesional delivery of anti-human antibodies. Nigra is to left of each image, striatum is to right. Scale bar, 1 mm.

(Hamilton, USA) mounted on a micromanipulator (World Precision Instruments, USA). To make each infusion, the stylet was removed and the prefilled infusion cannulae was inserted fully, up to the bend, through the indwelling cannula such that the ventral tip reached the site of transection. Infusions were made at 1 μL/min, allowing 2 mins diffusion time prior to slow removal of the infusion cannula and reocclusion of the indwelling cannula with the stylet. Use of clear polythene tubing allowed delivery of equal volumes of substance to be confirmed by eye.

Histology

Eleven days post-transection, animals given terminal anaesthesia (2 mL/kg intraperitoneal, Euthatal, Roche Meriaux, France) were perfused transcardially using 100 mL phosphate buffered saline (PBS) and then 300 mL 4% paraformaldehyde in PBS. Brains were dissected out and maintained overnight in the same fixative prior to immersing in cryoprotectant (30% sucrose, 0.1% sodium azide in PBS) until they sunk. Ten series of 40 μm-thick parasagittal sections were cut on a sledge microtome (Leica, UK) prior to processing various series either to visualize axons of the dopaminergic nigrostriatal tract or to establish the effects of treatments upon various non-neuronal cells or extracellular matrix molecules. A standard immunoperoxidase protocol was followed (Moon et al. 2000) using appropriate blocking serum (normal goat or donkey, Dako, UK) and the following primary antibodies: goat anti-human IgG (Fc specific, peroxidase conjugated, 1 : 200, Sigma, UK); rabbit antibodies against tyrosine hydroxylase (TH, 1 : 4,000, Jacques Boy Institut, France); rabbit antibodies against glial fibrillary acidic protein (GFAP, 1 : 10,000, Dako, UK); mouse monoclonal antibodies against complement receptor 3 (CR3) (clone MRC OX42, 1 : 200, Serotec, UK), against NG2 (clone D31-10, 1 : 4, gift of J. Levine), or mouse monoclonal antibodies CS56 (1 : 100, Sigma, UK). Control immunostaining was performed using appropriate concentrations of mouse IgM (Sigma, UK) or mouse IgG1 (Sigma, UK) in place of the primary antibody. Appropriate biotinylated secondary antibodies were used (horse anti-mouse, rat adsorbed, Vector, UK; goat anti-rabbit IgG, Dako, UK; rabbit anti-goat IgG, Sigma, UK) in conjunction with a streptavidin/biotinylated horseradish peroxidase kit (Dako, UK) with diaminobenzidine as the chromagen. Sections were mounted on presubbed (1% gelatin in PBS) glass slides, dehydrated in an ascending series of ethanols, cleared in xylene and coverslipped using DPX.

Analysis

Optical densitometry was used to assess the extent of gliosis at the lesion site, defined by immunoreactivities for GFAP, NG2, CR3 or CS56. High power (× 100) images of the lesion site were captured using a digital camera mounted on a light microscope (Leitz DMRB, Leica, UK) coupled to an IBM-compatible PC running an appropriate graphics package (Photoshop, Adobe). Images were saved as TIFF files without compression and exported to NIH image (Scion Image, release beta 3b, Scion Corp.). Lighting conditions and exposures were equal within capture sessions and variabilities due to regions of cavitation associated with near-zero pixel densities were corrected for mathematically. A one millimetre-square field of view including the lesion core was analysed. As these results were taken from three similar experiments, optical density data were normalized across sessions by reassigning the mean of the control IgG4 group from each experiment as 100% and scaling other data accordingly.

Catecholaminergic neurons, including dopaminergic neurons of the nigrostriatal tract, were visualized by immunostaining using antibodies against TH. Dopaminergic nigrostriatal axon growth within brain parenchyma, anterior to the lesion core, was quantified by counting at high magnification (× 400) the number of TH-immunoreactive processes crossing an imaginary line of length 3000 μm drawn perpendicular to the course of the original nigrostriatal tract and 500 μm anterior to the plane of transection. Counts were made in two medial sections from each animal. Group differences were assessed using analysis of variance (ANOVA) using a standard level of significance ($P < 0.05$).

Results

The following terminology will be used: 'axotomy + sham' refers to the group of animals that were given unilateral knife cut lesions of the nigrostriatal tract with sham infusions; 'axotomy + IgG4' refers to the group of animals that were given unilateral knife cut lesions and infusions of saline containing control human IgG4 antibodies and 'axotomy + antiTGFβ' refers to the group of animals that were given unilateral knife cut lesions and infusions of saline containing a

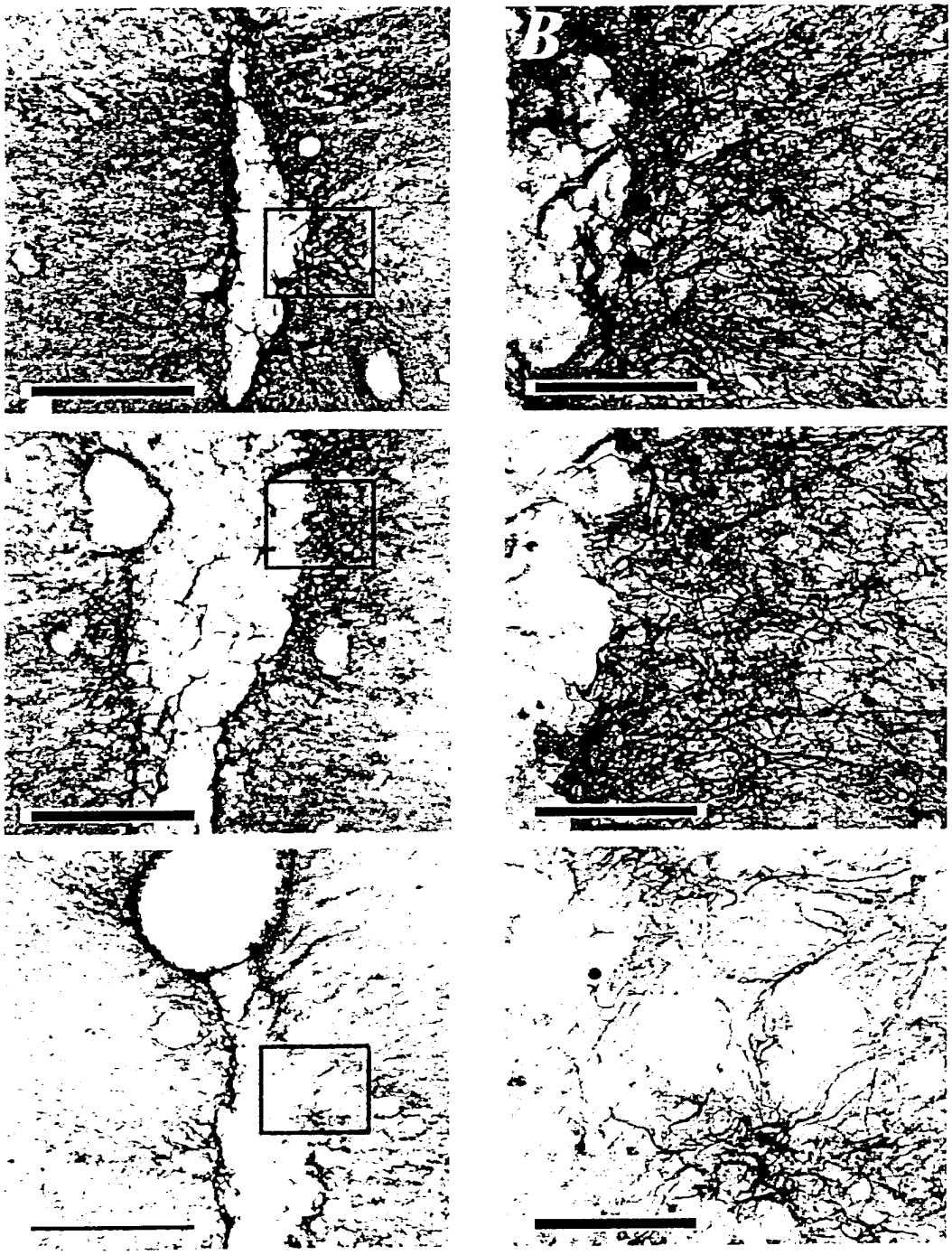


FIG. 3. GFAP-immunolabelling showing perilesional astrocytosis following (A–B) transection and sham infusion or (C–D) perilesional infusion of either control IgG4 antibodies or (E–F) antibodies against TGFβ1 and TGFβ2, examined 11 days postaxotomy. Astrocytosis was reduced following treatment with antibodies against TGFβ1 and TGFβ2. Nigra is to left of each image, striatum is to right. (A, C and E) Scale bar, 400 μm (B, D and F) Higher magnification view of rectangular area indicated in (A, C and E), respectively. Scale bar, 100 μm.

combination of antibodies to TGFβ1 and TGFβ2. It should be noted that for technical reasons, data from groups of animals treated with neurotrophins (see Table 1) were excluded from the analysis of gliosis and is only included where the response of dopaminergic nigral axons was examined. Names of these groups have not been abbreviated in the text.

Gross histological examination indicated a region of tissue damage incorporating both the site of axotomy and (except in 'axotomy + sham' animals) the site of infusion. These regions will be referred to collectively as the 'lesion core'. In 'axotomy + sham' animals this lesion core extended a mean distance of 300 μm anterior to the plane of transection, whereas

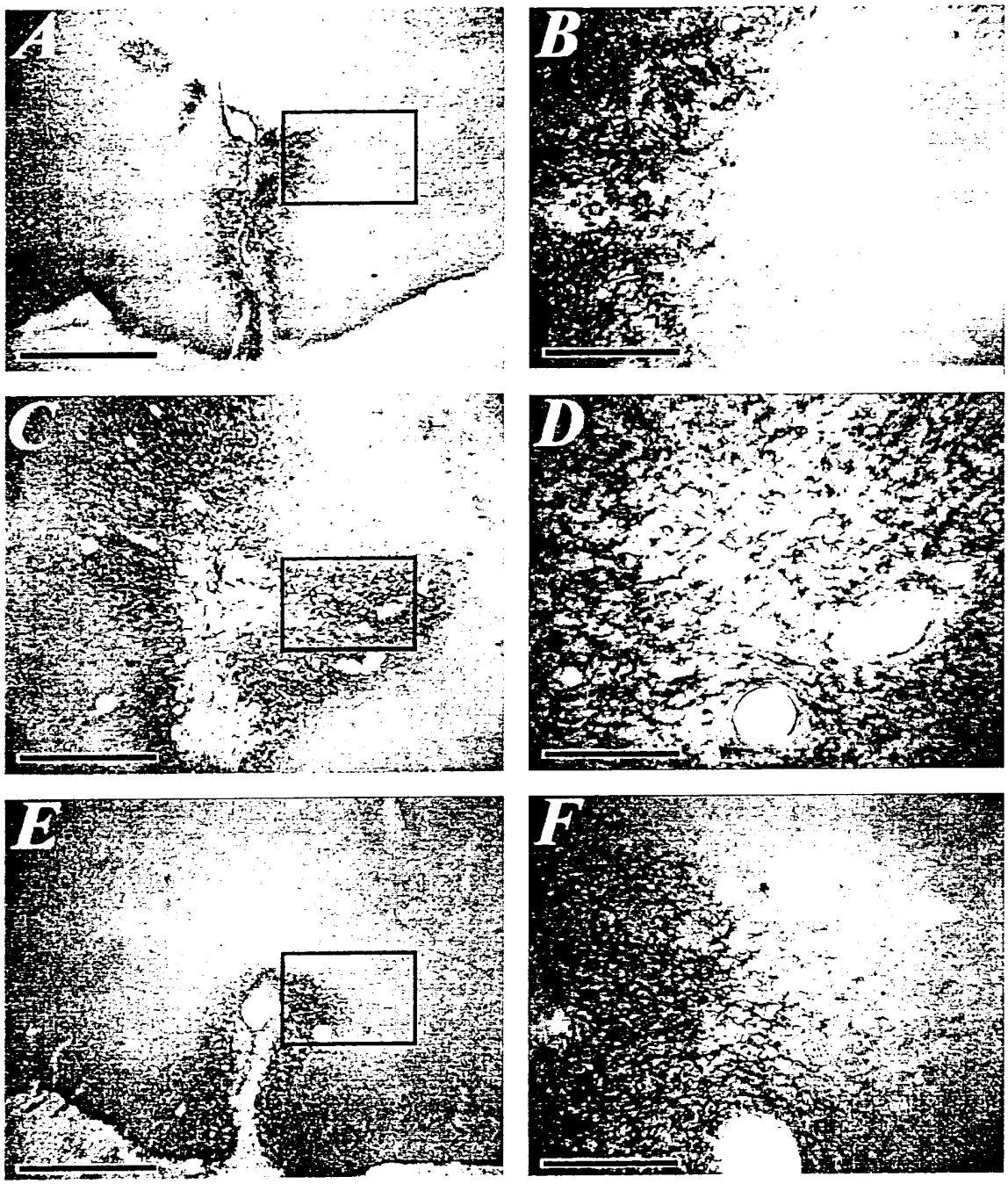


FIG. 4. NG2-immunolabelling for stellate adult oligodendrocyte progenitors following transection and (A–B) sham infusion or treatment with either (C–D) control IgG4 antibodies or (E–F) antibodies against TGFβ1 and TGFβ2, examined 11 days postaxotomy. Following transection, relative to sham controls, infusion of IgG4 control antibodies enhanced abundance of NG2-immunoreactivity. However, relative to IgG4 controls, following transection and treatment with antibodies against TGFβ1 and TGFβ2, NG2-immunoreactivity was attenuated to levels similar to that observed following transection with sham infusion. Nigra is to left of each image, striatum is to right. (A,C and E) Scale bar, 1000 μm (B, D and F) Higher magnification view of rectangular area indicated in (A, C and E), respectively. Scale bar, 250 μm.

in all other groups of animals this extended a mean distance of 1000 μm anterior to the plane of transection.

Immunostaining with anti-human antibodies

Eleven days post-transection (i.e. 1 day following the last infusion), to confirm the effective delivery of human control antibodies (IgG4) or human antibodies to TGFβ1 and TGFβ2 *in vivo*, anti-human antibodies were used to immunoperoxidase label one series of sections. Immunostaining demonstrated the presence of human antibodies within the core of lesion and within a 300-μm-thick rim immediately surrounding the lesion core in 'axotomy + IgG4' animals or 'axotomy + antiTGFβ' animals (Fig. 2). The spatial distribution of control and experimental antibodies appeared similar between groups and low magnification optical densitometry revealed

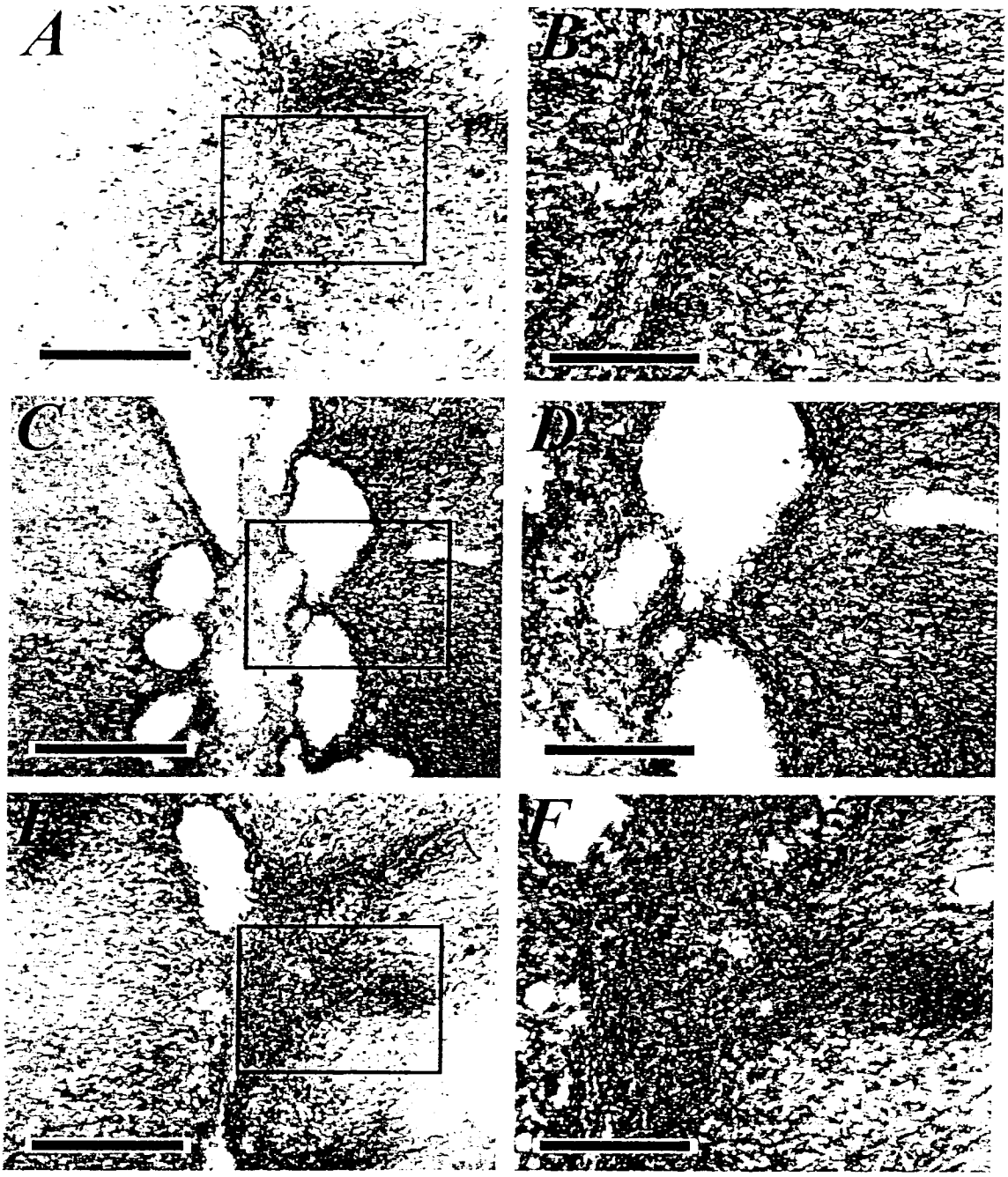


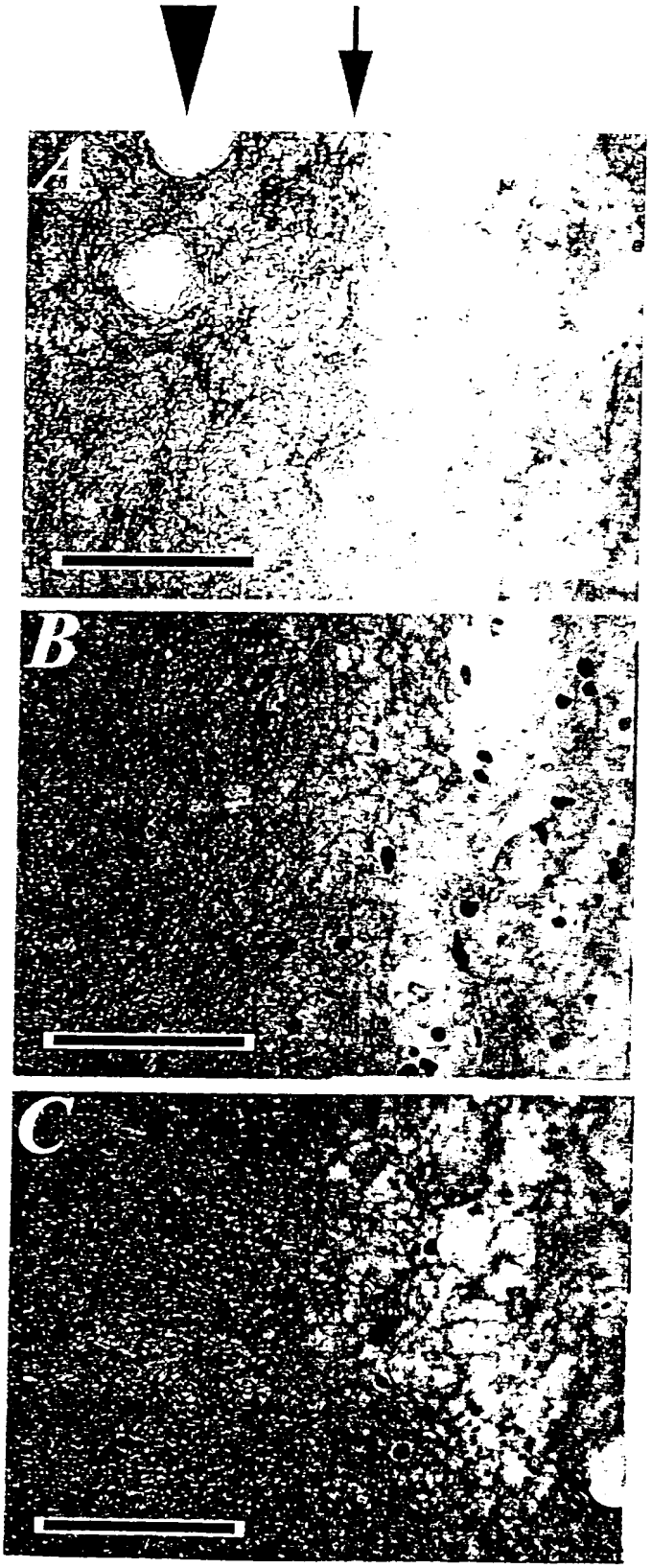
FIG. 5. CR3-immunolabelling for microglia and macrophages following transection and (A–B) sham infusion or treatment with either (C–D) control IgG4 antibodies or (E–F) antibodies against TGFβ1 and TGFβ2, examined 11 days postaxotomy. Following transection, relative to sham controls, infusion of IgG4 control antibodies or antibodies against TGFβ1 and TGFβ2 resulted in enhanced abundance of CR3-immunoreactivity. Nigra is to left of each image, striatum is to right. (A, C and E) Scale bar, 400 μm (B, D and F) Higher magnification view of rectangular area indicated in (A, C and E), respectively. Scale bar, 200 μm.

no differences between groups in the abundance of immunoreactivity ($t_8 = 0.12$, $P = 0.91$). Thus, both control and experimental antibodies were delivered effectively to the region in which reactive gliosis is observed.

Astrocyte response

The astrocytic response was examined using antibodies against GFAP. In all animals, 11 days post-transection, GFAP-immunoreactivity was associated with stellate cells throughout the ipsilateral hemisphere. In 'axotomy + sham' animals (Fig. 3A and B) or 'axotomy + IgG4' animals (Fig. 3C and D), GFAP-immunoreactivity was particularly intense in the lesion surround, walling the lesion core off completely with a dense network of cell bodies and hypertrophied processes mostly orientated perpendicular to the lesion border (Fig. 3E and F). GFAP-immunoreactivity was also present surrounding regions of cavitation. However, GFAP-immunoreactivity was

absent from the lesion core. The mean rostrocaudal length of the region lacking significant numbers of GFAP-immunoreactive cells was 100 μm.



In 'axotomy + antiTGFβ' animals, the extent of GFAP-immunoreactivity was reduced such that fewer astrocyte cell bodies and processes were present surrounding the lesion core, particularly within 250 μm of the lesion border (Fig. 3B). In many places, GFAP-immunoreactivity did not 'wall-off' the lesion core and appeared loose and discontinuous.

Analysis of data obtained by low magnification optical densitometry (Fig 7a) revealed a significant difference between groups ($F_{2,15} = 5.12$, $P = 0.023$) and *post hoc* comparisons demonstrated a significant difference between the anti-TGFβ and IgG4 groups (Tukey test, $P < 0.05$). This demonstrates that infusion of antibodies to $TGF\beta_1$ and $TGF\beta_2$ (relative to IgG4 control antibodies) results in reduced GFAP-immunoreactivity, assessed 11 days following transection

Adult oligodendrocyte precursor response

The response of adult, stellate oligodendrocyte progenitor cells was examined using antibodies against NG2 chondroitin sulphate proteoglycan (Nishiyama et al., 1999). In 'axotomy + sham' animals, the lesion core essentially lacked stellate NG2-immunoreactive cells although it contained NG2-immunoreactivity associated with blood vessels (Fig. 4A and B). The lesion core and, where present, small cavities, were surrounded by large numbers of stellate NG2-immunoreactive cells whose processes ran perpendicularly to the lesion border and formed a dense network of hypertrophic processes forming a continuous *glia limitans*-like structure, this often being less dense caudally. Thus, although cells with low NG2-immunoreactivity were present throughout the ipsilateral hemisphere, the region containing stellate cells intensely immunoreactive for NG2 were confined to within 250 μm of the lesion core.

In 'axotomy + IgG4' animals, a similar pattern of NG2-immunoreactivity was detected (Fig. 4C and D). However, as the conjoined site of transection and infusion was larger than following transection alone, the total amount of NG2-immunoreactivity was greater in these animals. However, in 'axotomy + antiTGFβ' animals, the extent of NG2-immunoreactivity was reduced such that fewer oligodendrocyte progenitor cell bodies and processes were present surrounding the lesion core, particularly tissue within 250 μm of the lesion border (Fig. 4E and F).

These observations were confirmed by analysis of variance of data obtained by low magnification optical densitometry (Fig. 7b) which indicated a significant difference between groups ($F_{2,15} = 9.91$, $P = 0.002$); *post hoc* analysis demonstrated a significant difference between the anti-TGFβ and IgG4 groups and between the IgG4 and sham infusion groups (Tukey test, $P < 0.05$). Thus, relative to 'axotomy + sham' controls, while repeated perilesional infusion of IgG4 control antibodies enhanced NG2 gliosis surrounding the site of axotomy, this was attenuated following repeated perilesional infusion of antibodies against anti-$TGF\beta_1$ and anti-$TGF\beta_2$.

Microglial and macrophage response

Antibodies against complement receptor 3 (CR3, clone MRC-OX42) were used to identify microglia and macrophages. Following

FIG. 6. CS56-immunolabelling for chondroitin sulphate glycosaminoglycans following transection and (A) sham infusion or treatment with either (B) control IgG4 antibodies or (C) antibodies against TGFβ1 and TGFβ2, examined 11 days postaxotomy. Arrow indicates level of transection, arrowhead indicates lesion surround. Scale bar, 100 μm.

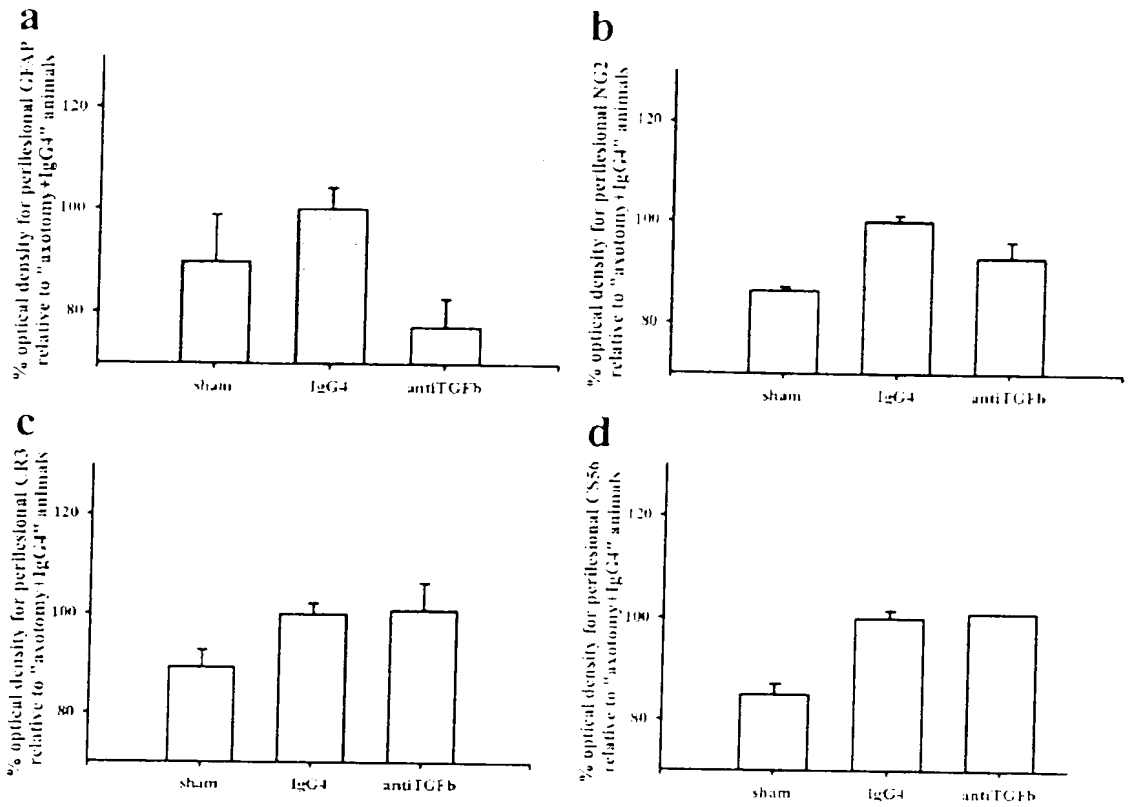


FIG. 7. Graphs showing normalized percentage optical densities ('axotomy + IgG4' animals taken as 100%), for perilesional immunoreactivities for (a) GFAP (b) NG2 (c) CR3 and (d) CS56. Graphs indicate means and standard errors of means. Relative to 'axotomy + IgG4' animals, both 'axotomy + sham' and 'axotomy + antiTGFβ' animals showed significantly reduced perilesional immunoreactivities for GFAP and NG2. Relative to 'axotomy + sham' animals, perilesional immunoreactivities for CR3 and CS56 were greater in 'axotomy + IgG4' and 'axotomy + antiTGFβ' animals.

transection with sham infusion, delicately ramified CR3-immunoreactive microglia were visible throughout the ipsilateral hemisphere. Stellate ameboid CR3-immunoreactive cells were essentially absent from the lesion core but ameboid CR3-immunoreactive cells were present in this region (Fig. 5A and B). However, stellate CR3-immunoreactive cells were present within 500 μm of the lesion borders and, where present, small cavities. However, the density of CR3-immunoreactive cell bodies and processes in the lesion surround varied significantly such that, whereas some regions were entirely surrounded by CR3-immunoreactivity, other regions contained less intense CR3-immunoreactive cell bodies and processes.

In 'axotomy + IgG4' animals, a similar pattern of CR3-immunoreactivity was detected (Fig. 5C and D). However, as the conjoined site of transection and infusion was larger than following transection alone, the total amount of CR3-immunoreactivity was greater in these animals and extended up to 1000 μm from lesion borders. This was also the case in 'axotomy + antiTGFβ' animals (Fig. 5E and F) and there were no apparent differences in CR3-immunoreactivity between these two groups.

These observations were confirmed by analysis of variance of data obtained by low magnification optical densitometry (Fig. 7c) indicated a significant differences between groups ($F_{2,15} = 4.55$, $P = 0.0.32$); *post hoc* analysis demonstrated a significant difference between the 'axotomy + sham' and both other groups (Tukey test, $P < 0.05$). There was no difference between the 'axotomy + IgG4' and 'axotomy + antiTGFβ' groups.

TABLE 2. Treatment and numbers of TH-immunoreactive axons

Treatment	Number of TH-immunoreactive axons counted 500 μm anterior to the site of transection (mean ± SEM)
Axotomy + sham	14.3 ± 6.2
Axotomy + saline + antiTGFβ$_1$ + antiTGFβ$_2$	19.6 ± 2.8
Axotomy + saline + IgG4 control antibodies	19.5 ± 3.9
Axotomy + saline + GDNF	16.7 ± 10.4
Axotomy + saline + GDNF + antiTGFβ$_1$ + antiTGFβ$_2$	12.0 ± 4.0
Axotomy + saline + bFGF + IL-1α + antiTGFβ$_1$ + antiTGFβ$_2$	18.0 ± 2.1

CS GAG assessed by immunostaining for CS56

Chondroitin sulphate glycosaminoglycans (CS GAG) were detected by immunostaining using the immunoglobulin-M (IgM) antibody, CS56, which recognizes epitopes predominantly present in both chondroitin-4 and chondroitin-6 sulphate glycosaminoglycans and to a lesser extent in both heparan- (Avnur & Geiger, 1984) and dermatan- sulphate glycosaminoglycans (Lips et al., 1995). Recent work suggests that CS56 does not recognize standard 0-, 4- or 6-sulphated units which comprise the backbone of chondroitin sulphate chains but rather atypical motifs distributed nonrandomly within

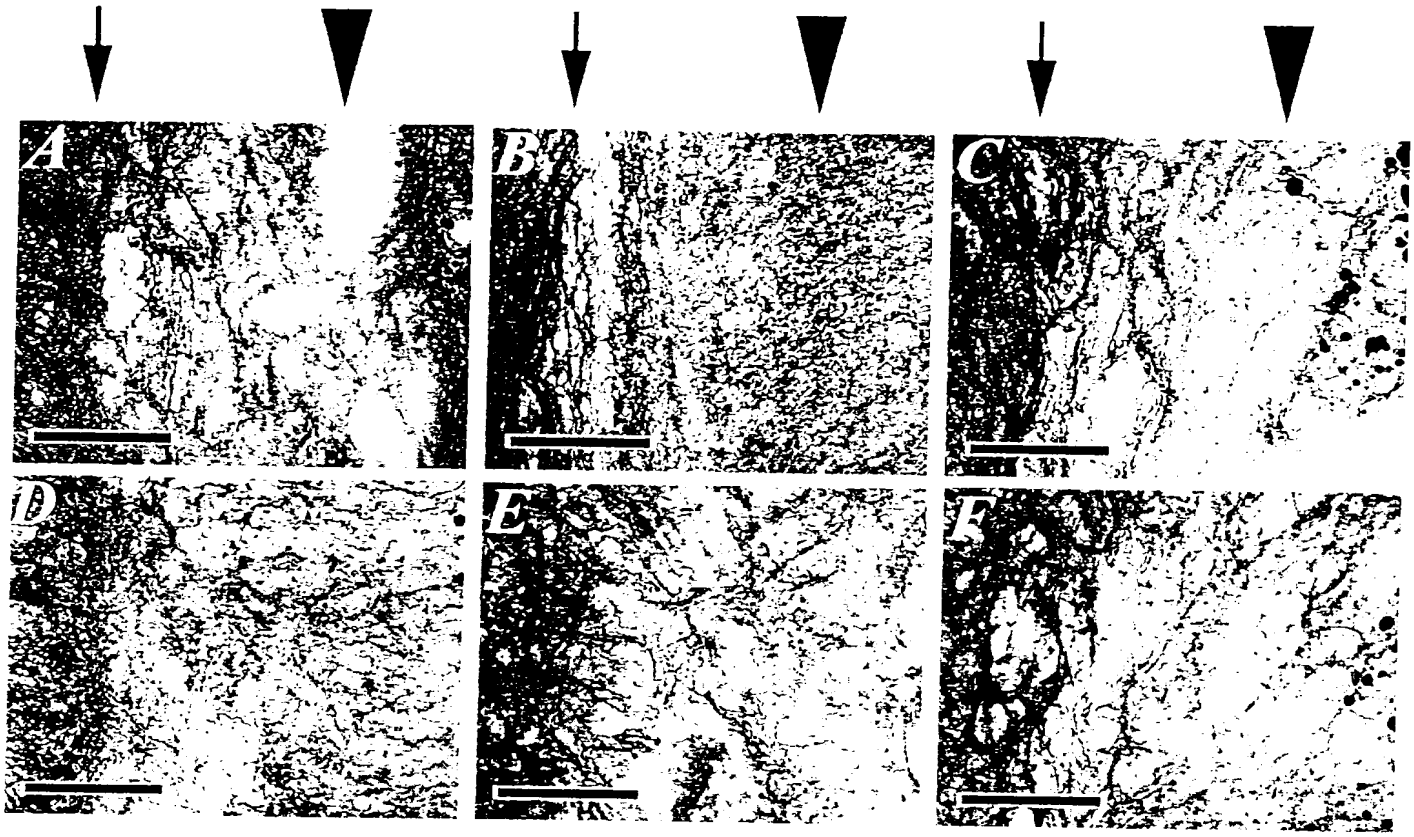


FIG. 8. Tyrosine hydroxylase-immunolabelling for catecholaminergic neurons including dopaminergic nigral axons 11 days following nigrostriatal transection and (A) sham infusion or treatment with (B) control IgG4 antibodies (C) GDNF, or antibodies against $TGF\beta_1$ and $TGF\beta_2$ either (D) alone or (E) in combination with GDNF, or (F) with both bFGF and IL-1α. No differences between groups were observed in the number of TH-reactive processes growing more than 500 μm anterior to the plane of transection. Balls of TH-immunoreactive debris were often observed. Nigra is to left of each image, striatum is to right. Arrow indicates level of transection, arrowhead indicates level of infusion. Scale bar, 250 μm.

these chains (Sorrell et al., 1993). Control immunostaining using mouse IgM antibodies was used to determine the level of nonspecific immunoreactivity associated with the staining protocol due in part to crossreactivity of mouse IgM antibodies with endogenous rat antibodies present in the acute lesion environment.

Examined 11 days post-transection, diffuse, extracellular CS56-immunoreactivity was present in 'axotomy + sham' animals in a 100-μm-thick halo surrounding and within the lesion core (Fig. 6A). In contrast, in 'axotomy + IgG4' (Fig. 6B) and 'axotomy + antiTGFβ' animals (Fig. 6C), diffuse, extracellular CS56-immunoreactivity was present within and up to 2000 μm from the lesion. CS56-immunoreactivity was absent from the lesion core in all animals.

Analysis of variance of data obtained by low magnification optical densitometry (Fig. 7d) revealed significant differences between groups ($F_{2,7} = 29.8$, $P = 0.002$) and *post hoc* comparisons (Tukey test, $P < 0.05$) demonstrated significant differences between 'axotomy + sham' and both other groups, indicating that infusion of substance enhances abundance of CS GAGs. There were no other differences between groups indicating that, relative to IgG4 control antibodies, a combination of antibodies to $TGF\beta_1$ and $TGF\beta_2$ did not reduce abundance of CS GAGs assessed 11 days post-transection.

Response of dopaminergic nigral axons

The response of dopaminergic nigrostriatal axons was visualized 11 days post-transection by immunostaining using antibodies against TH. In 'axotomy + sham' animals, the distal portion of the dopaminergic nigrostriatal tract degenerated with loss of innervation of the ipsilateral striatum and without spontaneous long distance axon regeneration. At this time, TH-immunoreactive debris was scarce. TH-immunoreactive fibres were observed sprouting ectopically within the lesion core, often being orientated parallel to the plane of transection, i.e. perpendicular to the original nigrostriatal tract (Fig. 8A). Axons were often fasciculated. TH-immunoreactive nigral axons were also observed bordering small cavities (where present) and ventral to brain parenchyma in meninges which had been penetrated during transection. Axons typically extended rostrally no more than 200 μm and did not regenerate beyond the lesion core.

In all other groups of animals, a similar pattern of axonal growth was observed, whether treated with control IgG4 antibodies (Fig. 8B) or GDNF (Fig. 8C) or with antibodies against $TGF\beta_1$ and $TGF\beta_2$ either alone (Fig. 8D) or in combination with GDNF (Fig. 8E) or with both bFGF and IL-1α (Fig. 8F). Thus, large numbers of TH-immunoreactive processes sprouted ectopically within the lesion, often being fasciculated. Many of these axons grew perpendicular to the orientation of the original nigrostriatal tract (i.e. parallel to the plane of transection). Again, TH-immunoreactive axon sprouts did not regenerate beyond the lesion core although in all groups, a small number of axons extended up to 800 μm within the lesion core anterior to the plane of transection.

Treatment with GDNF or with both bFGF and IL-1α (either alone or in combination with antibodies against $TGF\beta_1$ and $TGF\beta_2$) did not

visibly enhance growth of dopaminergic nigral axons anterior to the site of transection. Indeed, when assessed formally, there was no difference between groups in the number of dopaminergic nigral axons to grow 500 μm anterior to the plane of transection (Table 2; analysis of variance, $F_{5,26} = 0.35$, $P = 0.87$). This indicates that, assessed 11 days postinjury, relative to IgG4 controls, perilesional administration of antibodies against both TGFβ$_1$ and TGFβ$_2$, either alone or in combination with GDNF or bFGF with IL-1α, did not significantly enhance local sprouting of dopaminergic nigral axons within brain parenchyma beyond the lesion core.

Discussion

We have shown that, following unilateral nigrostriatal transection in the adult rat, treatment with a combination of antibodies to TGFβ$_1$ and TGFβ$_2$ reduces (but does not abolish) the response of astrocytes and NG2-immunoreactive adult oligodendrocyte progenitor cells, although not the response of microglia/macrophages or CS GAG. However, despite this reduction in gliosis, treatment with a combination of antibodies to TGFβ$_1$ and TGFβ$_2$ did not enhance the regeneration of cut dopaminergic nigral axons beyond the lesion core; in particular, cut dopaminergic nigral axons did not regenerate back to their original primary target, the ipsilateral striatum.

These results are consistent with previous experiments demonstrating that various TGFβ antagonists modulate aspects of scar formation *in vivo* (Logan et al., 1994; Nath et al., 1998; Logan et al., 1999a; Logan et al., 1999b). Our work similarly implies that TGFβ isoforms regulate gliosis associated with both astrocytes and NG2-immunoreactive oligodendrocyte progenitors, although further experiments are required to determine which, if either, isoform is the more important for regulation of the response on NG2 oligodendrocyte progenitor cells. The present failure to detect any effect upon microglia/macrophages of the combination of antibodies against TGFβ$_1$ and β$_2$ may reflect a cancelling out of activity by the two isoforms as, when administered alone following cortical injury in adult rats, whereas TGFβ2 reduces the microglial/macrophage response (Logan et al., 1999b), TGFβ1 increases it (Logan et al., 1994). Arguing against this possibility, however, is the observation that administration of the pan-TGFβ antagonist, decorin, reduces the microglia/macrophage response following cortical injury (Logan et al., 1999a).

We hypothesized that the reduction in gliosis might have been only partial because of the route of administration of antibodies, *viz.*, by repeated perilesional infusion, as the gliotic response to injury was potentiated by each intraparenchymal injection. To test this hypothesis, an additional experiment was performed (data not shown). Adult rats were given unilateral mechanical nigrostriatal lesions and transcranial cannulae were implanted transcranially allowing repeated intraventricular infusion of vehicle containing either IgG4 ($n = 6$) or the combination of antibodies against TGFβ$_1$ and TGFβ$_2$ ($n = 4$). However, no differences were found between groups in any measure of scar formation, nor in growth of axons beyond the plane of transection; these results likely reflect poor diffusion of adequate concentrations of antibodies from the ventricles to the site of transection (which breached the CNS–CSF barrier). Indeed, the control and experimental human antibodies were not detectable at the transection site by immunoperoxidase-labelling using anti-human antibodies. This study suggests that the intraventricular route may not be an effective route for delivery of these antibodies into distal lesion sites and leaves unresolved the question as to whether an alternative method of delivery of antagonists to TGFβ might result in a more pronounced reduction in gliosis.

Our results regarding the failure of cut dopaminergic nigral axons to grow through the region of reduced scar formation are consistent with unpublished data cited previously (Logan et al., 1994). This work suggested that attenuation of scar formation mediated by antibodies against TGFβ$_1$ occurs without concomitant increases in local sprouting or long distance axon regeneration 10 days following cortical stab injury in adult rats, as assessed by immunostaining for neurofilament or growth associated protein-43. It should be noted that in our study, effective delivery of human antibodies was confirmed by immunolabelling tissue (using anti-human secondary antibodies) 1 day following the last infusion of control and experimental antibodies. Our failure to detect an effect upon axons is, therefore, not a consequence of failed delivery. Successful delivery of antibodies is, of course, also confirmed by the effects upon gliosis that we have described.

The failure of cut dopaminergic nigral axons to grow through the region of reduced scar formation is probably due to the reduction in scar formation being partial rather than complete; complete abolition of scar formation may, thus, be required to induce substantial long distance CNS axon regeneration. In particular, we observed no significant reduction in perilesional abundance of CS GAGs, (assessed by immunolabelling using CS56 antibodies) that are known to limit growth *in vitro* and *in vivo*. Thus, anti-TGFβ-mediated down-regulation of astrocytosis (assessed using GFAP-immunolabelling) may not be sufficient to down-regulate levels of CS GAGs. Possible explanations include (i) independent regulation of CS GAGs and GFAP by astrocytes and (ii) continued synthesis of CS GAGs by other cell types (including NG2-immunoreactive cells). It is also possible that changes did occur in CS GAGs other than those recognized by the CS-56 antibody; at present it is not clear which CSPGs this antibody recognizes. These results emphasize that although GFAP is a good marker for astrocyte reactions to lesions, other markers for gliosis provide important additional information (e.g. by immunolabelling for NG2-immunoreactive cells or for CS GAGs).

In some cases, additional potential regeneration-enhancing molecules were delivered together with the antibodies against TGFβ$_1$ and TGFβ$_2$: glial cell line derived neurotrophic factor (GDNF) or the combination of basic fibroblast growth factor (bFGF) with interleukin-1α (IL-1α). These factors and their doses were selected on the basis of previous work conducted in laboratories including our own. First, GDNF enhances outgrowth of axons from dopaminergic nigral neurons *in vitro* and *in vivo* (Sinclair *et al.*, 1996; Wilby et al., 1999). Second, a combination of bFGF and IL-1α makes three dimensional cultures of astrocytes more permissive for ingrowth of neurites from neonatal rat dorsal root ganglion neurons (Fok-Seang et al., 1998). Further, as this effect can be blocked by TGFβ, one would predict that the combination of antibodies to TGFβ$_1$ and TGFβ$_2$ with bFGF and IL-1α might further enhance neurite penetration of regions of scar formation. However, disappointingly, repeated intraparenchymal administration of these factors, either alone or in combination with antibodies to TGFβ$_1$ and TGFβ$_2$, did not significantly enhance dopaminergic nigrostriatal axon growth through or beyond the lesion core. A failure to detect any growth may relate to the rather small numbers of animals used and additional experiments would be required to evaluate the significance of this negative result more rigorously. Nevertheless, in this small study, efforts to boost axon regeneration through the partially attenuated scar following administration of neurotrophins were not successful.

In conclusion, *in vivo* administration of antibodies against $TGF\beta_1$ and $TGF\beta_2$ significantly reduce gliosis induced by nigrostriatal transection in the adult rat. However, this partial reduction is not accompanied by any change in growth of cut dopaminergic nigral axons. In the future it will be important to evaluate alternative methods of delivery of these antibodies to achieve a more complete reduction in gliosis.

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Abbreviations

bFGF, basic fibroblast growth factor; CS GAG, chondroitin sulphate glycosaminoglycans; GDNF, Glial cell-line Derived Neurotrophic Factor; GFAP, Glial Fibrillary Acidic Protein; IL-1, Interleukin-1; TGF, Transforming Growth Factor β.

References

Asher, R.A., Morgenstern, D.A., Adcock, K.A., Rogers, J.H. & Fawcett, J.W. (1999) Versican is up-regulated in CNS injury and is a product of O-2A lineage cells. *Soc. Neurosci. Abstr.*, **25**, 750.

Asher, R.A., Morgenstern, D.A., Fidler, P.S., Adcock, K.A., Oohira, A., Braistead, J.E., Levine, J.M., Margolis, R.K., Rogers, J.H. & Fawcett, J.W. (2000) Neurocan is upregulated in injured brain and in cytokine-treated astrocytes. *J. Neurosci.*, **20**, 2427–2438.

Avnur, Z. & Geiger, G. (1984) Immunocytochemical localization of native chondroitin-sulfate in tissues and cultured cells using a specific monoclonal antibody. *Cell*, **38**, 811–822.

Baghdassarian-Chalaye, D., Tour-Delbauffe, D., Gavaret, J.M. & Pierre, M. (1993) Effects of transforming growth factor β1 on the extracellular matrix and cytoskeleton of cultured astrocytes. *Glia*, **7**, 193–202.

Brecknell, J.E., Dunnett, S.B. & Fawcett, J.W. (1995) A quantitative study of cell death in the substantia nigra following a mechanical lesion of the medial forebrain bundle. *Neuroscience*, **64**, 219–227.

Brecknell, J.E. & Fawcett, J.W. (1996) Axonal regeneration. *Biol. Rev.*, **71**, 227–255.

Flanders, K., Ludecke, G., Renzing, J., Hamm, C., Cissel, D. & Unsicker, K. (1993) Effects of TGFβs and bFGF on astroglial cell growth and gene expression *in vitro*. *Mol. Cell. Neurosci.*, **4**, 406–417.

Fok-Seang, J., DiProspero, N.A., Meiners, S., Muir, E. & Fawcett, J.W. (1998) Cytokine-induced changes in the ability of astrocytes to support migration of oligodendrocyte precursors and axon growth. *Eur. J. Neurosci.*, **10**, 2400–2415.

Kneglstein, K., Rufer, M., Suter-Crazzolara, C. & Unsicker, K. (1995) Neural functions of the transforming growth factors beta. *Int. J. Dev. Neurosci.*, **13**, 301–315.

Lips, K., Stichel, C.C. & Müller, H.W. (1995) Restricted appearance of tenascin and chondroitin sulphate proteoglycans after transection and sprouting of adult rat postcommissural fornix. *J. Neurocytol.*, **24**, 449–464.

Logan, A., Baird, A. & Berry, M. (1999a) Decorin attenuates gliotic scar formation in the rat cerebral hemispheres. *Exp. Neurol.*, **159**, 504–510.

Logan, A. & Berry, M. (1993) Transforming growth factor beta 1 and basic fibroblast growth factor in the injured CNS. *Trends Pharmacol. Sci.*, **14**, 397–343.

Logan, A., Frautschy, S.A., Gonzalez, A.M., Sporn, M.B. & Baird, A. (1994) Effects of transforming growth factor beta 1 on scar production in the injured central nervous system. *Eur. J. Neurosci.*, **6**, 355–363.

Logan, A., Green, J., Hunter, A., Jackson, R. & Berry, M. (1999b) Inhibition of glial scarring in the injured rat brain by a recombinant human monoclonal antibody to transforming growth factor beta 2. *Eur. J. Neurosci.*, **11**, 2367–2374.

McCartney-Francis, N.L. & Wahl, S.M. (1994) Transforming growth factor beta: a matter of life and death. *J. Leukocyte Biol.*, **55**, 401–409.

Moon, L.D.F., Asher, R.A., Rhodes, K.E. & Fawcett, J.W. (2001) Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC. *Nature Neurosci.*, **4**, 465–466.

Moon, L.D.F., Brecknell, J.E., Franklin, R.J., Dunnett, S.B. & Fawcett, J.W. (2000) Robust regeneration of CNS axons through a track depleted of CNS glia. *Exp. Neurol.*, **161**, 49–66.

Nath, R.K., Kwon, B., Mackinnon, S.E., Jensen, J.N., Reznik, S. & Boutros, S. (1998) Antibody to transforming growth factor beta reduces collagen production in injured peripheral nerve. *Plastic Reconstructive Surg.*, **102**, 1100–1108.

Nishiyama, A., Chang, A. & Trapp, B. (1999) NG2+ glial cells: a novel glial cell population in the adult brain. *J. Neuropath. Exp. Neurol.*, **58**, 1113–1124.

Paxinos, G. & Watson, C. (1986) *The Rat Brain in Stereotaxic Coordinates*. Academic Press, Sydney.

Raivich, G., Bohatschek, M., Kloss, C.U.A., Werner, A., Jones, L.J. & Kreutzberg, G.W. (1999) Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Res. Rev.*, **30**, 77–105.

Schnädelbach, O., Mandl, C. & Faissner, A. (1998) Expression of DSD-1 PG in primary neural and glial-derived cultures, upregulation by TGF-beta and implications for cell–substrate interactions of the glial cell line oli-neu. *Glia*, **23**, 99–119.

Sinclair, S.R., Svendsen, C.N., Torres, E.M., Martin, D., Fawcett, J.W. & Dunnett, S.B. (1996) GDNF enhances dopaminergic cell survival and fiber outgrowth in embryonic nigral grafts. *Neuroreport*, **7**, 2547–2552.

Smith, G.M. & Hale, J.H. (1997) Macrophage/microglia regulation of astrocyte tenascin: synergistic action of transforming growth factor beta and basic fibroblast growth factor. *J. Neurosci.*, **17**, 9624–9633.

Sorrell, J.M., Carrino, D.A. & Caplan, A.I. (1993) Structural domains in chondroitin sulfate identified by anti-chondroitin sulfate monoclonal antibodies. Immunosequencing of chondroitin sulfates. *Matrix*, **13**, 351–361.

Varga, J., Rosenbloom, J. & Jiminez, S.A. (1987) Transforming growth factor beta causes a persistent increase in steady-state amounts for type I and type II collagen and fibronectin mRNAs in normal dermal human fibroblasts. *J. Biochem.*, **297**, 597–604.

Wilby, M.W., Sinclair, S.R., Muir, E.M., Zeitlow, R., Adcock, K.A., Horellou, P., Rogers, J.H., Dunnett, S.B. & Fawcett, J.W. (1999) A glial cell line-derived neurotrophic factor-secreting clone of the schwann cell line SCTM41 enhances survival and fiber outgrowth from embryonic nigral neurons grafted to the striatum and to the lesioned substantia nigra. *J. Neurosci.*, **19**, 2301–2312.